

# Ecdysteroid titers and developmental expression of ecdysteroid-regulated genes during metamorphosis of the yellow fever mosquito, *Aedes aegypti* (Diptera: Culicidae)

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## Abstract

Ecdysteroid titers and expression profiles of ecdysone-regulated genes were determined during the last instar larval and during the pupal stages of *Aedes aegypti* (Diptera: Culicidae). Three peaks of ecdysteroids occurring at approximately 24, 30–33 and 45–48 h after ecdysis to the fourth instar larval stage were detected. In the pupa, a large peak of ecdysteroids occurred between 6 and 12 h after ecdysis to the pupal stage. A small rise in ecdysteroids was also detected at the end of the pupal stage. Quantitative reverse transcriptase polymerase chain reaction analyses of the expression of ecdysone receptors and ecdysone-regulated genes showed that the peaks of expression of most of these genes coincided with the rise in ecdysteroid levels during the last larval and pupal stages. In the last larval stage, ecdysteroid titers and mRNA expression profiles of ecdysone-regulated genes are similar to those observed for *Drosophila melanogaster*. However, in the early pupal stage, both ecdysteroid titers and the expression of ecdysone-regulated genes are somewhat different from those observed in *D. melanogaster*, probably because the duration of the pupal stage in *D. melanogaster* is 84 h while in *Ae. aegypti* the duration is only 48 h. These data which describe the relationship between ecdysteroid titers and mRNA levels of *Ae. aegypti* ecdysteroid-regulated genes lay a solid foundation for future studies on the hormonal regulation of development in mosquitoes.

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**Keywords:** Hormone action; Gene expression; Molting; Development

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## 1. Introduction

The yellow fever mosquito, *Aedes aegypti* (Diptera: Culicidae) is an important insect vector that transmits pathogens that are causative agents for yellow fever, dengue and other serious diseases. There is considerable information available concerning the physiological and biochemical basis of feeding, osmotic regulation and reproduction of *Ae. aegypti* (Hagedorn et al., 1985; Bownes, 1986; Hagedorn, 1996; Raikhel et al., 2002). However, except for a few recent studies (Lan and Grier, 2004; Nishiura et al., 2005) information about the endocrine regulation of development during the immature stages of mosquitoes is lacking; much must be inferred

from studies on model insects such as *Drosophila melanogaster* and *Manduca sexta*.

Studies using several model insects including *Bombyx mori*, *Hyalophora cecropia*, *M. sexta* and *D. melanogaster* have identified key hormones and elucidated their roles in molting and metamorphosis (Riddiford, 1993; Gilbert et al., 2000; Riddiford et al., 2000; Rybczynski, 2005). The neuropeptide prothoracicotropic hormone (PTTH) is produced by neurosecretory cells in the brain and released into the hemolymph from its neurohemal organ. PTTH stimulates the prothoracic glands (PGs) to produce ecdysteroid precursors to the molting hormone, typically 20-hydroxyecdysone (20E). In the presence of the sesquiterpene juvenile hormone (JH), 20E triggers a larval molt. In the last larval instar, in the absence of JH, a small peak of 20E has been shown to be responsible for a change from larval to pupal commitment. A second peak of 20E triggers apolysis

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(the separation of the old larval cuticle from the epidermis) followed by the formation of new pupal cuticle. It is after apolysis, during the pharate pupal stage that metamorphosis from the larva to the pupa takes place. In the pupal stage, a large peak of 20E, in turn, triggers apolysis and the initiation of adult formation. Metamorphosis from the pupa to the adult occurs in the pharate adult stage, while the adult insect is still encased in its pupal cuticle.

Ecdysteroids are present in eggs and larval, pupal and adult stages of mosquitoes (Hagedorn et al., 1975; Redfern, 1982; Borovsky et al., 1986; Russo and Westbrook, 1986; Whisenton et al., 1989; Jenkins et al., 1992; Lan and Grier, 2004). Ecdysteroid fluctuations during the mosquito's last larval instar have been reported previously. However, results are somewhat contradictory. Jenkins et al. (1992) reported a single peak in the hemolymph at 27 h post-ecdysis to the last instar; Fournet et al. (1995) found two large peaks, at 58 and 64 h post-ecdysis; and Lan and Grier (2004) detected a small commitment peak at 14–28 h, and based on ecdysone receptor (EcR) gene transcription results, predicted that a second and larger peak occurs at 46–66 h post-ecdysis. In mosquito pupae, Whisenton et al. (1989) and Fournet et al. (1995) both reported the presence of one large ecdysteroid peak. Thus, additional information as to the precise fluctuations of ecdysteroid titers during the entire fourth instar larval and during pupal developmental periods of *Ae. aegypti* is needed.

Twenty hydroxyecdysone transduces its signals by binding to a heterodimer of two nuclear receptors, EcR and ultraspiracle (USP) (Koelle et al., 1991; Yao et al., 1992, 1993; Palli et al., 2005). EcR–USP heterodimers mediate 20E effects through the regulation of transcription of genes involved in the 20E signaling cascade. Genes such as E75, E74 and hormone receptor 3 (HR3) are direct targets of the EcR–USP heterodimer. While other genes such as hormone receptor 38 (HR38), seven-up and ftz transcription factor 1 (ftz-f1) are regulated indirectly through 20E-induced transcription factors. In the current study, we measured ecdysteroid titers during the 4th instar larval stage and in the pupal stage. In addition, we determined the developmental profile of mRNAs of ecdysone receptors (EcR and USP), ecdysone-inducible early genes, E75 (three isoforms A, B and C) and E74, mRNA profiles were also monitored for *Aedes* hormone receptor 3 (AHR3) and three other nuclear receptors, *Aedes* hormone receptor 38 (AHR38), seven-up (AaSvp) and Aaftz-f1, receptors that are known to be involved in ecdysone signal transduction. The mRNA expression profiles of these ecdysone-regulated genes were compared with ecdysteroid titer fluctuations. The data presented here show that in the last larval instar, the ecdysteroid titers and mRNA expression profiles for ecdysteroid-regulated genes correlate well and are similar to those that have been reported for *D. melanogaster*. However, in the early pupal stage, both ecdysteroid titers and the expression of ecdysone-regulated genes are quite different from those that have been observed in *D. melanogaster*.

## 2. Materials and methods

### 2.1. Mosquito rearing and staging

Mosquito larvae (*Ae. aegypti*, Waco strain) were reared under a photoperiodic regime of 16:8 h (L:D) at a temperature of  $27 \pm 1$  °C, on a diet of bovine liver powder solution (MP Biochemicals, LLC, Aurora, OH). Newly molted fourth instar larvae with white heads were collected and divided into two groups based on their body weight and reared in separate pans. These larvae were sampled every 3 h until the pupal molt. Similarly, newly molted pupae with white heads were separated and sampled at 6 h intervals.

### 2.2. Determination of ecdysteroid titers

Staged insects were homogenized individually in a 1.5 ml microcentrifuge tube that contained 250 µl of ice-cold 75% aqueous methanol. Homogenized samples were centrifuged at 13,000g and 4 °C. Supernatants were transferred to 6 × 50 mm borosilicate glass tubes that were then placed in crushed ice. Precipitates were resuspended in an additional 100 µl of 75% aqueous methanol, vortexed, placed on ice for 30 min and centrifuged as above. Supernatant washes were added to their respective 6 × 50 mm tubes. An enzyme immunoassay (EIA) was used to measure sample ecdysteroid concentrations (Kingan, 1989; Gelman et al., 2002). Briefly, the EIA is performed in a 96-well microtiter plate and is based upon the competition between ecdysteroid (in standard or sample) and a known amount of peroxidase-labeled conjugated ecdysone for the ecdysteroid antiserum that has been bound to the IGG-coated wells. After several washes, the addition of TMB substrate (developed a blue color) followed by phosphoric acid (1 M) produced a yellow color. Absorbance was measured at 450 nm. The linear range of the assay is 0.5–40 pg. The ecdysteroid antiserum used in the EIA has a high affinity for ecdysone (E), 20E, makisterone A, 20,26-dihydroxyecdysone, 26-hydroxyecdysone and 3-dehydroecdysone (Kingan, 1989; T.G. Kingan, personal communication), but does not detect polar conjugates (Gelman et al., 2005).

### 2.3. RNA isolation and cDNA synthesis

Staged larvae and pupae were homogenized in TRI reagent (Molecular Research Center Inc., Cincinnati, OH) followed by RNA isolation according to the manufacturer's instructions. Total RNA concentration and purity were assessed using Genequant (Pharmacia, Piscataway, NJ). A total RNA volume equivalent to 5 µg was treated with DNase I (Ambion, Austin, TX) in a 25 µl total reaction volume following the manufacturer's protocol. cDNA was synthesized by reverse transcription using 1 µg of RNA and iScript cDNA synthesis kit (Biorad Laboratories, Hercules, CA) in a 20 µl reaction volume.

## 2.4. Quantitative real-time PCR (qRT-PCR)

The relative expression of selected genes was assessed by real-time PCR performed on a MyiQ single color real-time PCR detection system (Biorad Laboratories, Hercules, CA). qRT-PCR was performed using 1/40th of the volume of the cDNA with a PCR mastermix containing 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 0.2 mM of each dNTP (dATP, dCTP, dTTP and dGTP), 3 mM MgCl<sub>2</sub>, 1 unit of platinum *Taq* polymerase (Invitrogen Life Technologies, Carlsbad, CA), 1 × SYBR green I (Molecular Probes Inc., Eugene, OR) and 20 nM fluorescein. Normalization was performed using primers specific for S7 ribosomal protein mRNA. Primers for determining the developmental profiles of mRNA were designed based on the published sequence data deposited in Genbank (Table 1).

## 2.5. Statistical analysis

Analysis of variance was performed using SAS version 9.0 software (SAS Institute Inc., Cary, NC, USA) to test

Table 1  
Primers used in the QRT-PCR. Primer name, primer sequence and the reference are shown

Gene	Primer sequence (5'–3')	Reference
AaS7RP	f-ACCGCCGTCTACGATGCCA r-ATGGTGGTCTGCTGGTTCTT	
AaECRA	f-CCGTTACGGTTTACGCTAGTG r-TAGCCATTGGACATGGTGGA	(Wang et al., 2002)
AaECRB	f-GCAGGGTTGACATCAAGTATC r-CTAATCGCACGTTCTGCTTCA	(Cho et al., 1995; Wang et al., 2002)
AaUSPA	f-TACGTGACAACGTGTCCGAGCG r-TGTATTTCGTACCGCGTCCGTCG	(Kapitskaya et al., 1996)
AaUSPB	f-GCGGACGCCATGTTTCAATG r-GACCGGTCTTACACATTGAGC	(Kapitskaya et al., 1996)
AHR3	f-GTGTGCGGCGACAAGTCGTC r-TCGACCGAATTTGACCGCGTC	(Kapitskaya et al., 2000)
AHR38	f-GCCCGGTCAAAACGAAC r-TGCGCCTCCGAGTTGTA	(Zhu et al., 2000)
AaE74	f-CAGTAACAATCCCGCCAACT r-TGTTGGCTCATGACGCTTAG	(Sun et al., 2002)
AaE75A	f-CTCAGTCGCAAAACACCGGTCA r-TGTAAACTCTTTTGCGGCGCCGA	(Pierceall et al., 1999)
AaE75B	f-AGGAGCCCGCCAGTAACGAGAA r-TGTTGCTGCTGCTGCTACCGCT	(Pierceall et al., 1999)
AaE75C	f-CCTCAATCAGGCGCCGATGTCA r-CGGTTTGTTTCAAGGCTTTGCA	(Pierceall et al., 1999)
AaSvp	f-AGCATAGCTTGGCACCGAGCGC f-GCGAAGGTTGCAAATCG r-ATTTCCGGGCCAGTCAA	(Miura et al., 2002)
Aaftz-fl	f-TGAAGGTGGACGACCAGATGAA r-TGTTCTGCAGCTCGTTGAAGTG	(Li et al., 2000; Zhu et al., 2003)

for statistical differences among treatments ( $\alpha = 0.05$ ). Pair-wise comparisons were made using the protected least-squares difference (LSD) method.

## 3. Results

### 3.1. Ecdysteroid titer fluctuations during the fourth instar larval stage

Under our rearing conditions (See Materials and Methods), the duration of the 4th instar larval stage was approximately 51 h. Whole-body ecdysteroid titers were determined at 18 time points during this instar. In preliminary studies, titers of samples collected at nine time points (at 6 h intervals) were determined. Since considerable variation was observed in these data, in subsequent experiments, larvae were separated based on body weight and smaller larvae (mostly males) sampled at 3 h intervals were used. The latter sampling procedure provided more consistent results. Whole body ecdysteroid levels in final instar larvae showed three peaks of ecdysteroid, at 24, 30–33 and 45 h after ecdysis to the final larval instar (AEFL) (Fig. 1). Ecdysteroid levels were low (5–10 pg/larva) until 15 h AEFL. At 18 h AEFL, the level of ecdysteroids began to rise and the titer reached 68.7 pg/larva at 24 h AEFL. After a brief decline, the level of ecdysteroids increased dramatically and reached a maximum of 179.4 pg/larva at 30 h AEFL. These high levels were maintained through 33 h AEFL. Thus, ecdysteroid levels were significantly higher in the second peak as compared to the first. In addition, the duration of the smaller first peak was shorter (approximately 6 h) than the duration of the second (approximately 12 h). Ecdysteroid levels rose again between 39 and 45 h AEFL and peaked at approximately 117 pg/larva prior to the larval–pupal ecdysis.

### 3.2. Ecdysteroid titers during the pupal stage

Under our rearing conditions, the duration of the pupal stage was approximately 48 h. A large peak of ecdysteroid was observed soon after ecdysis to the pupal stage (AEP) (Fig. 2); maximum levels were attained at 6–12 h AEP. The level of ecdysteroid then decreased and remained low throughout the pupal stage except for a small increase at the end of this stage just prior to the pupal–adult ecdysis.

### 3.3. Gene expression profiles during the fourth instar larval stage

The levels of mRNA for both isoforms of EcR (AaEcRA and AaEcRB) were low at the beginning of the fourth instar and then started to increase at 6 h AEFL (Fig. 3). AaEcRA mRNA levels exhibited three peaks of activity, the first, a broad peak between 3 and 30 h, the second between 30 and 45 h and the third between 45 and 51 h. The last two peaks occurred at approximately the same time as

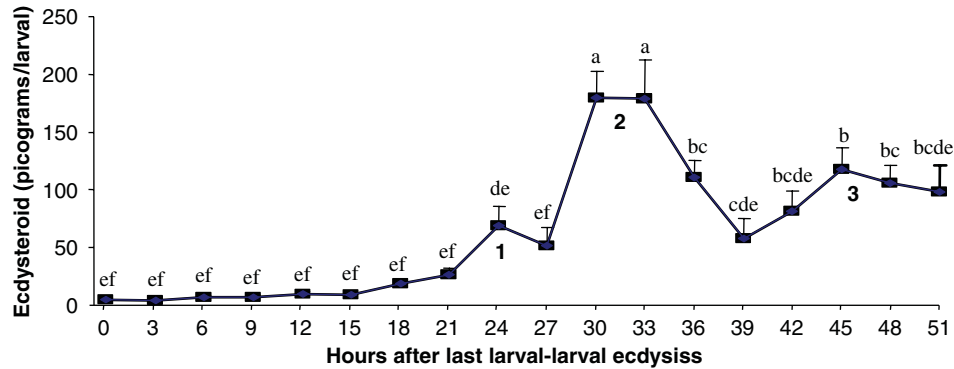


Fig. 1. Ecdysteroid fluctuations during the last larval instar of *Ae. aegypti*. For the determination of ecdysteroid titers, supernatants from whole body extracts of staged, individual larvae were assayed for ecdysteroids using an enzyme immunoassay (EIA). Ecdysteroid levels (expressed in pg 20E equivalents) were determined in 7–35 individual larvae at each time point, and the mean  $\pm$  SE are shown. Means with the same letter are not significantly different ( $\alpha \leq 0.05$ ; ANOVA). Peak numbers are shown below the line graph.

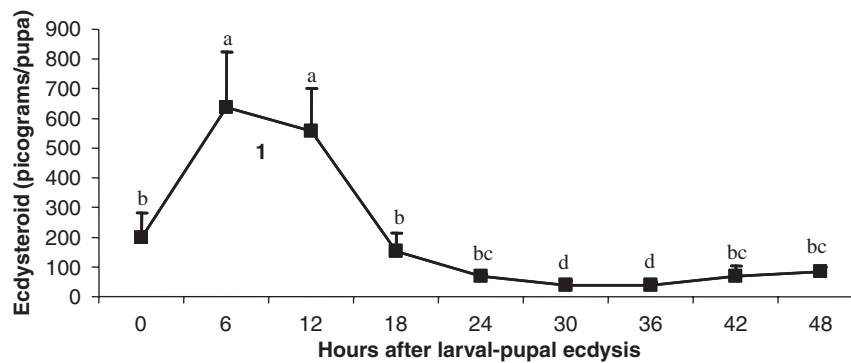


Fig. 2. Fluctuations in ecdysteroid levels during the pupal stage of *Ae. aegypti*. At 6h intervals until adult eclosion, newly molted pupae identified by the white color of the head were sacrificed, extracted and whole body ecdysteroid titers were determined by EIA. Each time point represents the mean  $\pm$  SE for 5–10 separate determinations. Ecdysteroid titers are expressed as picograms 20E equivalents per pupa. Statistical analysis was performed as described in the Materials and Methods section. Means with the same letter are not significantly different ( $\alpha \leq 0.05$ ; ANOVA).

the second and third peaks, respectively, observed for ecdysteroid. AaEcRB mRNA levels, while peaking between 3 and 12 h, only exhibited one additional peak which occurred at the end of the last larval instar, i.e., between 39 and 51 h, a peak that followed the second and coincided with the third ecdysteroid peak (Fig. 3A). AaUSP isoforms, AaUSPA and AaUSPB, showed patterns of expression in the last instar that were considerably different from each other (Fig. 3A). The mRNA levels for AaUSPA exhibited two peaks of expression, one at 0 h and the second at 36–48 h, whereas AaUSPB mRNA levels were very irregular and exhibited four peaks, the first between 6 and 18 h, the second between 30 and 36 h, the third between 36 and 45 h and the last between 45 and 51 h (Fig. 3A). Thus, the second AaUSPA peak of expression followed the second peak of ecdysteroid and began slightly before the third peak; the second and third AaUSPB peaks of expression coincided with the second ecdysteroid peak and the fourth AaUSPB peak coincided with the third ecdysteroid peak.

The temporal expression patterns for mRNAs of the three AaE75 isoforms, AaE75A, AaE75B and AaE75C,

showed three distinct patterns (Fig. 3B). AaE75A mRNA exhibited two peaks, a broad peak between 12 and 39 h and a second, rather high peak, between 39 and 51 h, the latter occurring at the end of the instar at approximately the same time as the last ecdysteroid peak. AaE75B also exhibited two peaks, but the span of these two coincided with the time frame for peak 1 of AaE75A, and AaE75B levels decreased toward the end of the instar. Importantly, peaks one and two of AaE75B expression coincided with ecdysteroid titer peaks one and two. AaE75C mRNA levels were low during the first 3 h after ecdysis; thereafter, three small peaks, one at 6 h, the second at 15 h and the third at 36 h AEFL were observed. A final, relatively large peak occurred at 48 h AEFL, a peak that coincided with the third ecdysteroid peak. AaE74 mRNA levels were relatively high at the time of ecdysis to the last instar (Fig. 3B). Levels decreased and remained low until 33 h AEFL, at which time they gradually increased producing a large bimodal peak between 33 and 51 h AEFL. The first part of the peak coincided with the second ecdysteroid peak and the last part of the peak with the third ecdysteroid peak (Fig. 3B).



The mRNA level of AHR3 began to rise at 21 h AEFL and reached maximum titer at 27 h AEFL (Fig. 3C). Then levels dropped and these low levels were maintained through the rest of the instar. Thus, the levels of mRNA for AHR3 were above basal levels for the same time period as ecdysteroid titers were above their basal levels. AHR38 mRNA levels were relatively high in the newly molted last instar larva, decreased dramatically by 3 h AEFL and remained low until 33 h AEFL except for a small rise at 24 h AEFL that coincided with the first ecdysteroid peak (Fig. 3C). The third and major peak for AHR38 occurred between 33 and 51 h AEFL, at the end of the

instar, approximately at the same time as the third ecdysteroid peak. The mRNA levels of AaSvp showed four peaks of expression, occurring at 18, 27, 39 and 51 h AEFL (Fig. 3C). Peak 2 occurred slightly later than the first small ecdysteroid peak, and again, titers were high at the end of the instar, peaks 3 and 4 coinciding with ecdysteroid peaks 2 and 3, respectively. The Aaftz-f1 mRNA expression pattern showed a single peak at 42 h AEFL, just after the second and just before the third ecdysteroid peak, shortly before the larval–pupal ecdysis (Fig. 3C). The level of Aaftz-f1 mRNA was low during the rest of the instar.

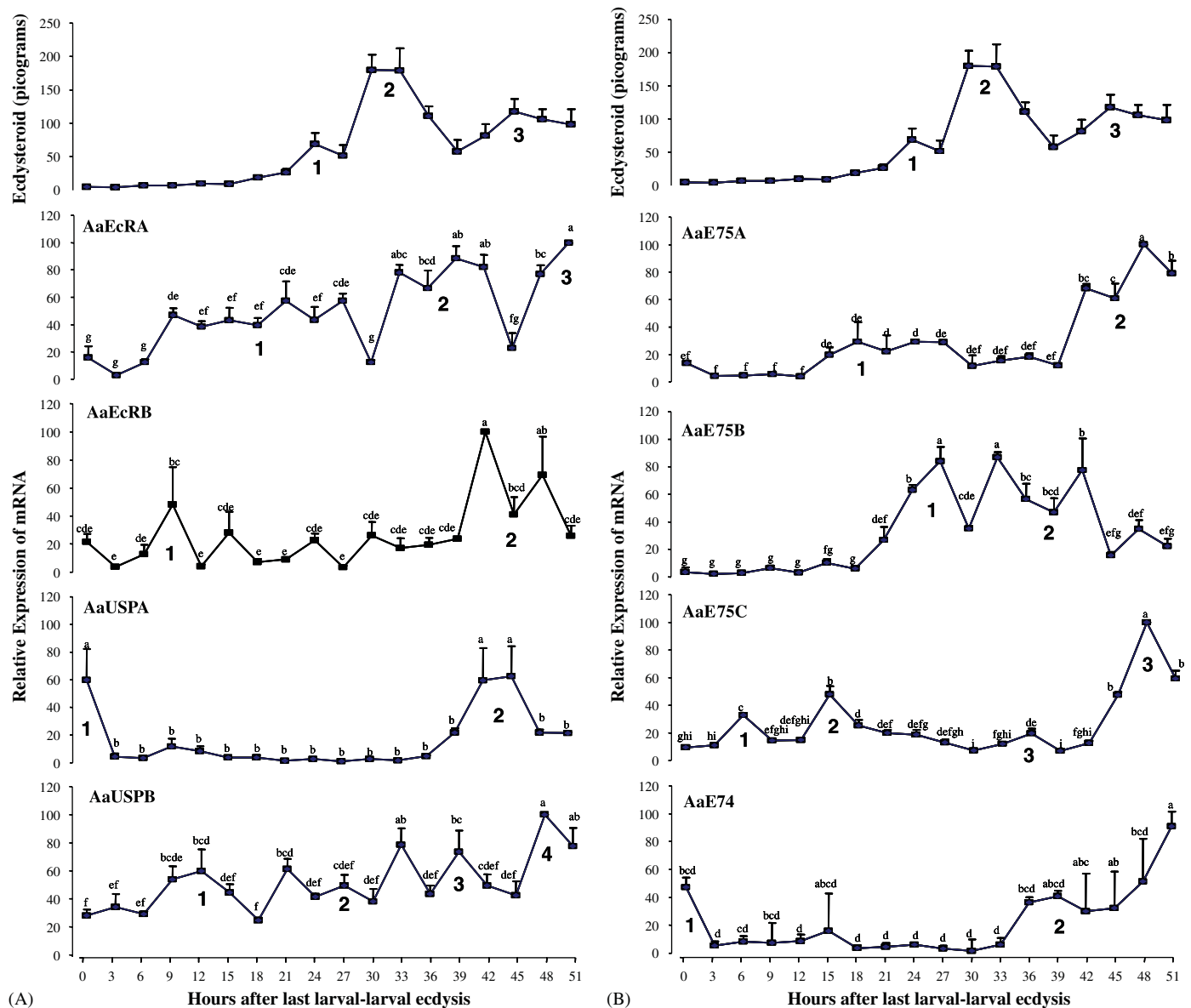


Fig. 3. mRNA expression profiles for ecdysone-regulated genes compared to fluctuations in ecdysteroid titer during the final larval instar of *Ae. aegypti*. Relative quantities of mRNA for ecdysone receptor isoforms AaEcRA and AaEcRB, ultraspiracle isoforms, AaUSPA and AaUSPB (A); ecdysone inducible genes AaE75 isoforms, AaE75A, AaE75B and AaE75C, and AaE74 (B) and early late gene AHR3, late genes AHR38, Aaftz-f1 and AaSvp (C) are compared to ecdysteroid levels during the final larval instar. Newly molted larvae were collected and sampled every 3 h until pupation. For each time point, RNA was isolated from six larvae. The level of mRNA was quantified by real-time PCR using SYBR green I for detection. Expression levels were normalized using S7RP ribosomal RNA as a standard. Relative expression was calculated by setting the maximum expression value at 100. Statistical analysis was performed as described in the Materials and Methods section. Means with the same letter are not significantly different ( $\alpha \leq 0.05$ ; ANOVA).

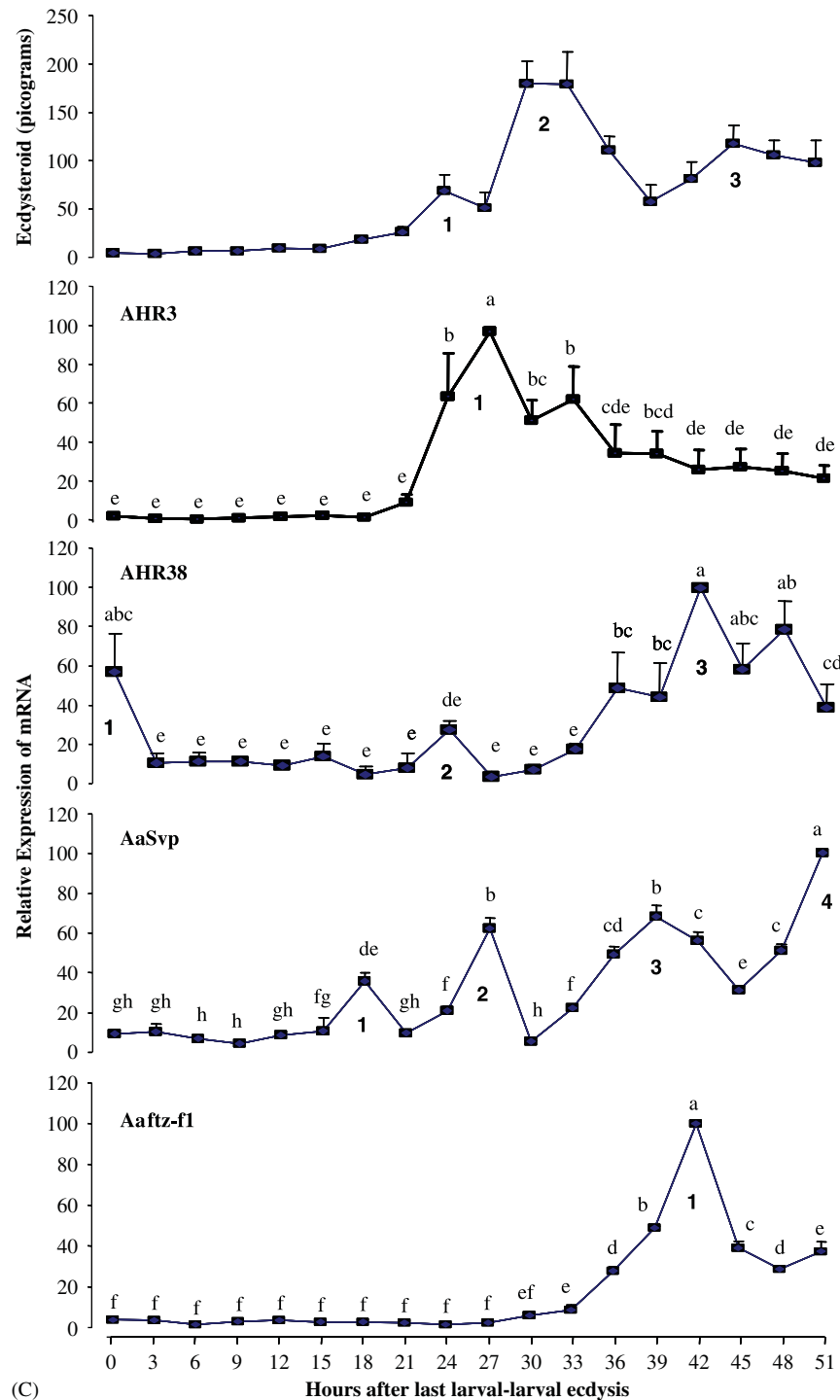


Fig. 3. (Continued)

### 3.4. Gene expression profiles during pupal stage

Isoforms of AaEcR, AaEcRA and AaEcRB, showed distinct expression patterns during the pupal stage. AaEcRA mRNA increased as ecdysteroid titers decreased and reached peak levels by 27 h AEP (Fig. 4A). In contrast, AaEcRB mRNA levels showed a major peak (#2) of expression that coincided with the single peak of ecdyster-

oid observed at the beginning of the pupal stage (Fig. 4A). The second peak of both isoforms of AaUSP expression also coincided with the pupal ecdysteroid peak (Fig. 4A). AaUSPA mRNA levels showed three additional peaks of expression, small peaks at 18 and 36 h and a larger one at 48 h. In contrast AaUSPB isoform mRNA levels remained low at the end of the pupal stage, although there was a small broad peak observed between 27 and 48 h.

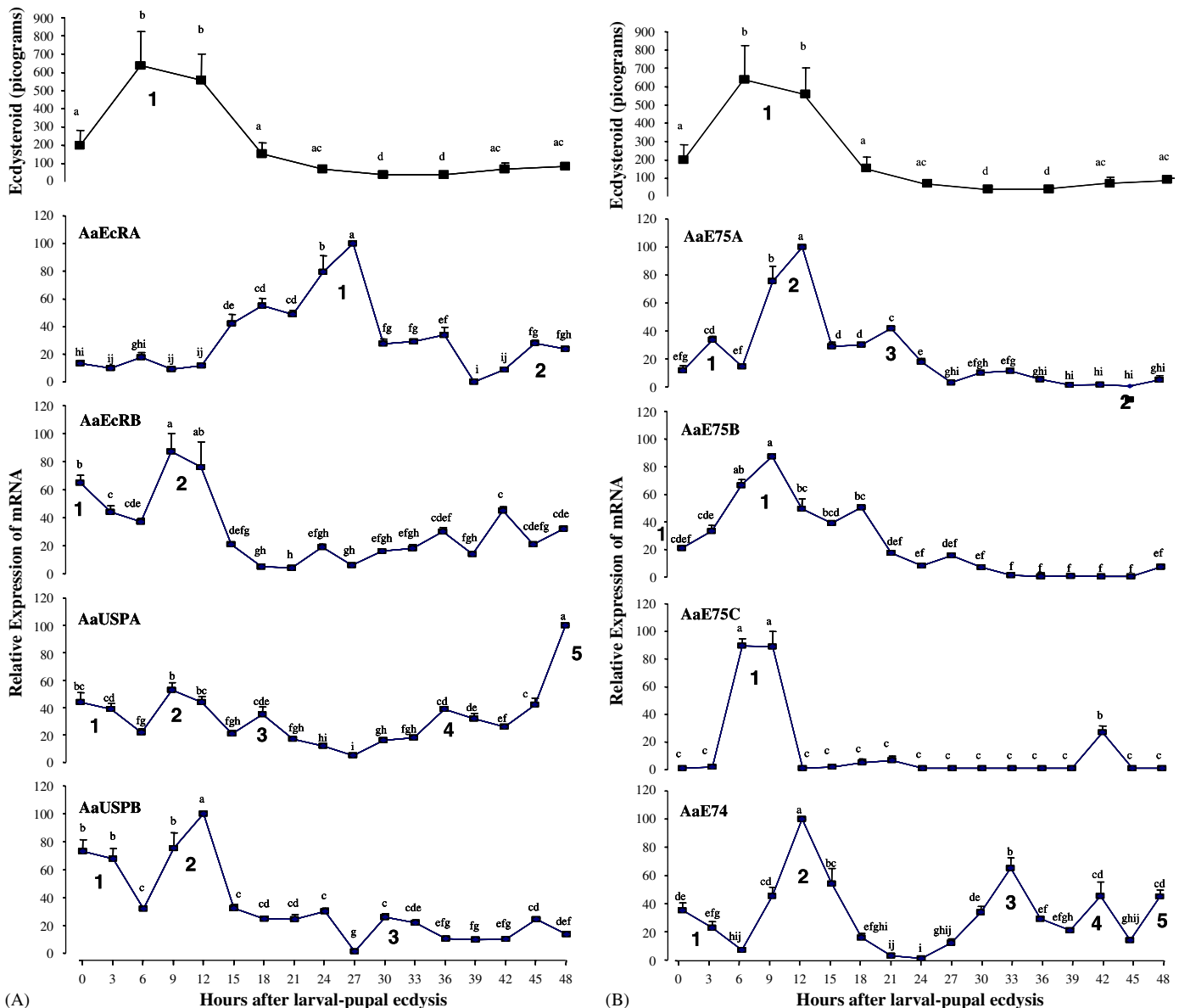


Fig. 4. mRNA expression profiles for ecdysone-regulated genes compared to fluctuations in ecdysteroid titer during the pupal stage of *Ae. aegypti*. Relative quantities of mRNA for ecdysone receptor isoforms AaEcRA and AaEcRB, ultraspiracle isoforms, AaUSPA and AaUSPB (A), ecdysone-inducible gene AaE75 isoforms, AaE75A, AaE75B and AaE75C, and AaE74 (B) and early late gene AHR3, late genes AHR38, Aaftz-f1 and AaSvp (C) are compared to whole body ecdysteroid levels in pupae. Newly molted pupae were collected and sampled every 3 h until pupation. For each time point RNA was isolated from six pupae. The levels of mRNA were quantified by a real-time PCR reaction using SYBR green I for detection as described in the legend to Fig. 3. Statistical analysis was performed as described in the Materials and Methods section. Means with the same letter are not significantly different ( $\alpha \leq 0.05$ ; ANOVA).

All three E75 isoforms, AaE75A and AaE75B and AaE75C, were prominently expressed during the initial few hours of the pupal stage, a time when ecdysteroid levels were high (Fig. 4B). AaE75A and AaE75B mRNA levels remained low during the rest of the pupal stage. AaE75C mRNA, however, showed a second small peak of expression at the end of the pupal stage. AaE74 mRNA levels, too, were elevated (peak at 12 h) when ecdysteroid titers were high and three additional peaks (at 33, 42 and 48 h) were observed later in the instar (Fig. 4B).

Four similar peaks of activity associated with AHR3 and AHR38 messenger RNA levels were observed (Fig. 4C). The first two peaks of these messenger RNAs coincided with the large pupal ecdysteroid peak. The mRNA levels of AaSvp exhibited four peaks in close proximity, the first two coinciding with the pupal ecdysteroid peak (Fig. 4C). In contrast, Aaftz-f1 mRNA levels were low during the first third of the pupal stage when ecdysteroid titers were high (Fig. 4C). Levels did not increase until 50% of the pupal stage and then three peaks (at 27, 36 and 45 h AEP) were observed, the largest being peak 2.

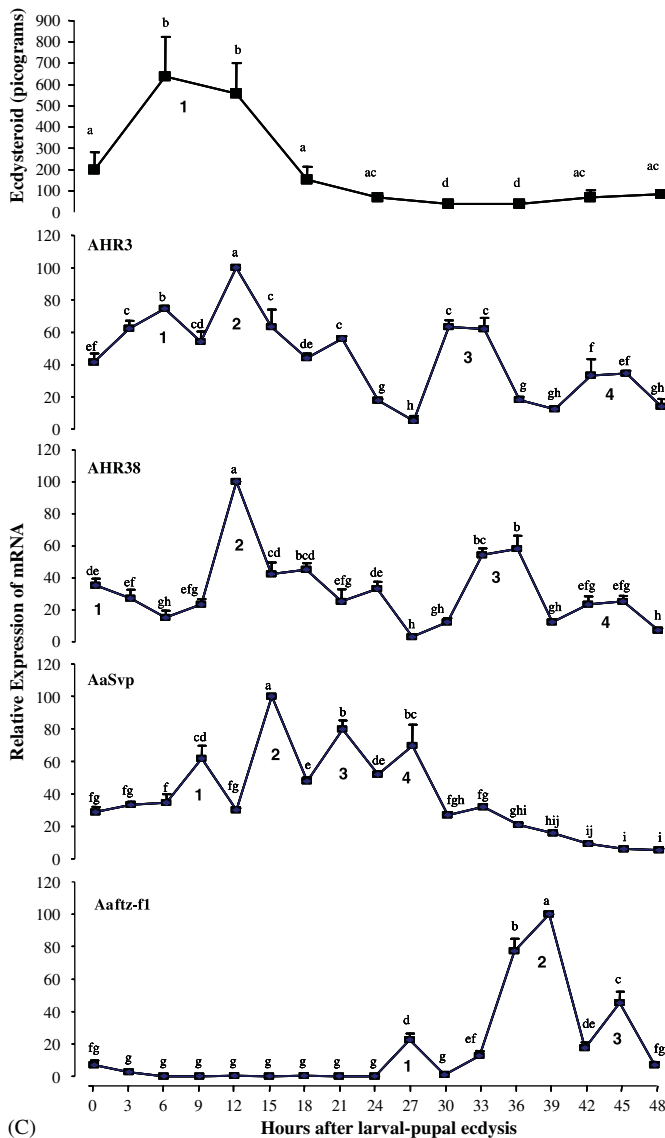


Fig. 4. (Continued)

In Fig. 5, the expression patterns of all of the genes of interest can be easily compared with each other and with ecdysteroid titer fluctuations during the fourth instar larval and pupal stages.

#### 4. Discussion

In these studies, the precise fluctuations of ecdysteroid titers in last instar larval and in pupal stages of *Ae. aegypti* have been determined. Three peaks of ecdysteroids were detected during the final larval instar of *Ae. aegypti*. The last peak probably begins in the pharate pupal stage when the new pupa is still encased in the old larval cuticle. This peak continues into the pupal stage, reaching maximum levels by 6–9 h after pupal ecdysis, and it is likely that it triggers the pupal–adult apolysis. Previously, Jenkins et al. (1992) measured ecdysteroid levels in hemolymph and in thoracic and abdominal tissues during the final larval instar

of *Ae. aegypti*. However, ecdysteroid levels were only titrated for the first 36 h (the duration of the instar was 36–52 h), and a single ecdysteroid peak, probably the pre-molt ecdysteroid peak, was observed at 27 h AEFL. Fournet et al. (1995) also measured ecdysteroid fluctuations in last instar larvae and in pupae to determine the effect of insect growth regulators (IGRs) on ecdysteroidogenesis. They reported two peaks of ecdysteroid during the final larval instar, one at 58 h and a second at 64 h AEFL, and a single large ecdysteroid peak during the pupal stage. Since the peaks occurred so late in the last instar, and since the duration of the last instar in males is shorter than in females (54 and 72 h AEFL, respectively), it is possible that the observed first and second peaks could represent the pre-molt ecdysteroid peak in males and females, respectively, rather than the commitment and pre-molt ecdysteroid peaks.

In a more recent study, Lan and Grier (2004) measured last instar larval ecdysteroid titers as part of an investigation to determine the time of pupal commitment. Two peaks of ecdysteroid were detected during the final larval instar, the first, a small peak that occurred at 18–24 h AEFL (results suggested that it was the commitment peak), and the second, based on EcR transcript titer data rather than direct measurement of ecdysteroid levels, a large peak that occurred at 46–66 h (the pre-molt ecdysteroid peak) AEFL. In our study, ecdysteroid titers were determined primarily in males at 3 h intervals throughout the fourth larval instar. We confirmed the presence of a commitment peak at 24 h (also reported by Lan and Grier, 2004) and detected a pre-molt ecdysteroid peak at 30–33 h, the latter earlier than the pre-molt ecdysteroid peak reported by these researchers. Differences in the timing of the ecdysteroid peaks among the three studies were probably due to differences in rearing temperatures, in populations of mosquitoes and in the relative percentages of males and females in the samples. During the pupal stage, we observed one large peak at the beginning of the stage as did Fournet et al. (1995) and Whisenton et al. (1989).

Since the objective of our study was to correlate ecdysteroid levels to gene expression profiles, it was important that we measure ecdysteroid fluctuations in our system. Using relative size as a parameter, we selected what we believed to be mainly males, and determined ecdysteroid levels at very short intervals, i.e., 3 h. In so doing, we used a more synchronous sample than other researchers and resolved some of the discrepancies arising from earlier studies in which pupation occurred between 36 and 52 h AEFL (Jenkins et al., 1992) or 54 and 72 h AEFL for males and females, respectively (Lan and Grier, 2004). The staging strategy followed in this study resulted in 90% pupation occurring between 48 and 52 h AEFL. In addition, pigmentation of developing pupal trumpets (Nishiura, 2002) was also used when staging pharate pupae at 45, 48 and 51 h AEFL.

Expression of ecdysone receptors, transcription factors and other ecdysone-regulated genes was compared with the



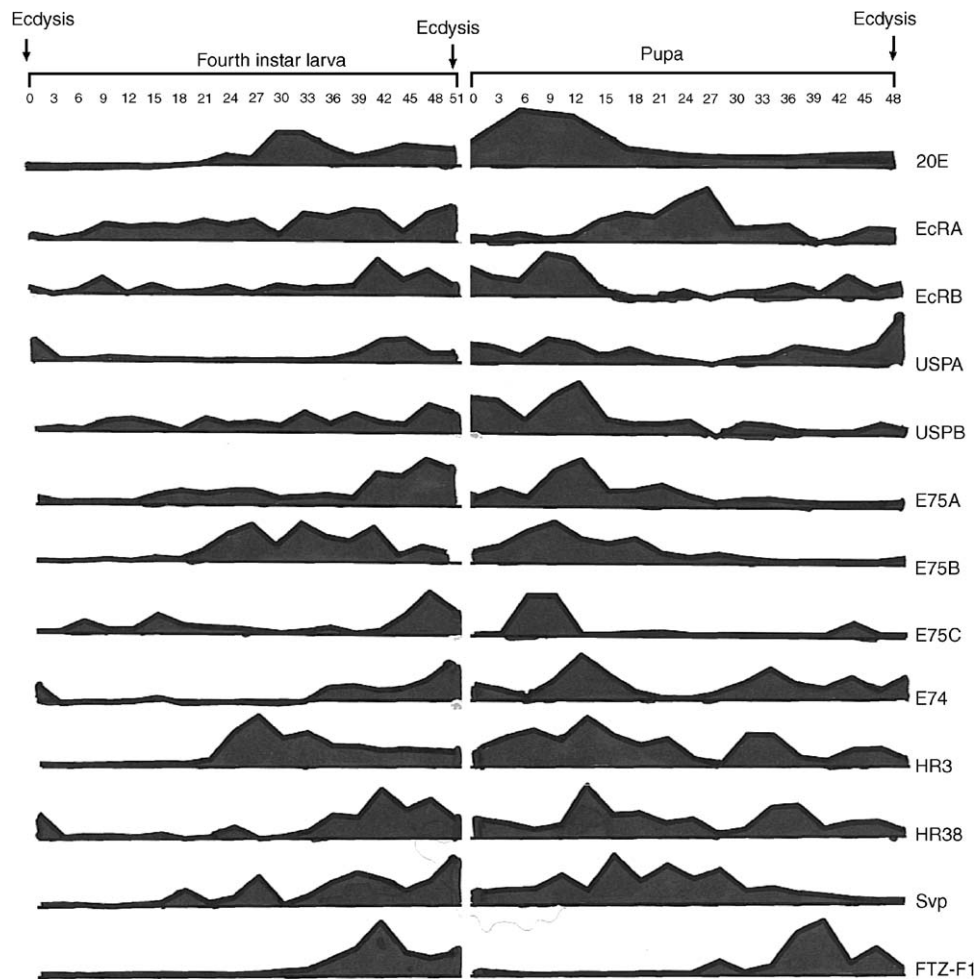


Fig. 5. Comparison summary of edysteroid titers and expression of ecdysone-regulated genes during the last larval instar and pupal stage of *Ae. aegypti*.

timing of ecdysteroid peaks. In most cases, expression correlates well with ecdysteroid titer fluctuations in last instar larval and pupal stages. Thus, in the larva, messenger RNA expression of all of the genes studied is high at the end of the instar and typically peaks of expression coincide with either or both of ecdysteroid peaks 2 and 3. In some cases, gene expression is relatively high at the time of the commitment peak of ecdysteroid (peak #1), i.e., for AaEcRA, AaE75A, AaE75B, AHR3, AHR38, and AaSvp. For the EcR gene, Lan and Grier (2004) reported a similar broad peak in expression at the time of the ecdysteroid commitment peak, and based on EcR expression concluded that there was a second peak, the pre-molt ecdysteroid peak, at the end of the instar. By sampling at 3 h intervals, we detected two peaks at the end of the instar, the first of which we believe triggers larval–pupal apolysis and the second of which may be the beginning of the pupal peak which, in turn, triggers pupal–adult apolysis. It is important to note that the EIA measures total ecdysteroid so that it is likely that ecdysteroids other than E (the immediate precursor to 20E) and 20E (physiologically active ecdysteroids) are present in the ecdysteroid peaks, including the third peak at the end of the larval instar.

However, it is often the ecdysteroid conjugates, inactivation products of E and 20E metabolism, that inflate the peaks (Gelman and Woods, 1986; Warren and Gilbert, 1986; Gelman et al., 1988), and as mentioned earlier, the antibody used in our EIA does not detect these conjugates. It is also likely that the increase in ecdysteroid during peak #3 is required for metamorphic changes occurring in the pharate pupal stage.

Using midguts dissected from fourth instar larvae, Nishiura et al. (2005) examined the expression of seven of the genes for which we report results here, AHR3, AaE75B, AaUSPB, AaUSPA, AaEcRB, Aaftz-f1 and AaE75A. The first two genes exhibited increased expression during the last 75% of the instar, and the last four genes showed increased expression during the last 25–50% of the instar. AaUSPB expression levels were constant during the instar, and in relative terms, AaEcRB expression levels tended to be 10 to 100 times higher during the entire instar than the expression levels of the other genes. Although measured in different samples, whole body (this study) and midgut (Nishiura et al., 2005), and although the duration of the last larval instar was 1.4–1.67 times longer (68–80 h) under the rearing conditions used by Nishiura

et al. (2005) than under our rearing conditions (48–52 h), gene expression in the two studies followed similar patterns.

For the most part, the expression patterns reported here for *Ae. aegypti* were similar to those reported for *D. melanogaster*. In *D. melanogaster*, there is a rise in ecdysteroid titer at the end of the last larval stage and a concomitant expression of most of the ecdysone-regulated genes. These increases continue as the puparium is formed and for a number of hours thereafter, triggering larval–pupal apolysis, which, in *Drosophila*, occurs at approximately 6 h post-puparium formation (Fechtel et al., 1989). A second small increase in both ecdysteroid titers and expression of ecdysone-regulated genes was observed at the end of the puparium stage (see Fig. 2 in King-Jones and Thummel, 2005) in the pharate pupal fly. Since *Ae. aegypti* larvae undergo metamorphosis and become pupae directly (there is no puparium stage in the mosquito), it may be that the third ecdysteroid peak accompanied by increased levels of ecdysone-regulated gene expression in the mosquito 4th instar larva corresponds to similar increases observed at the end of the puparium stage in *D. melanogaster*. Just after larval–pupal ecdysis, expression patterns were somewhat different in the two species. In *Drosophila* EcRB, E75A and E75B were not expressed while in *Ae. aegypti*, these genes were expressed, and in the fly, EcRA was expressed at a higher level than what we observed for *Ae. aegypti*. Expression of E75C and ftz-f1 were similar in young pupae of the two species. Differences in expression patterns as well as in the timing of the pupal ecdysteroid peak (9–12 h in *A. aegypti* and 24–72 h in *D. melanogaster*; Handler, 1982; Riddiford, 1993) are probably related to differences in the duration of the pupal/pharate adult stage which in *D. melanogaster* is 84 h, but in *A. aegypti* is only 48 h. The biological implications of these differences and the specific function(s) of ecdysone receptors and ecdysone-regulated transcription factors during mosquito development will be the subject of future investigations.

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